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ABSTRACT

Eighty-eight adenovirus (Ad) isolates and associated clinical data were collected from walk-in patients with influenza-like illness in Egypt (13 from Cairo, 75 from Alexandria) during routine influenza surveillance from 1999 through 2002. Ad diversity has not been well characterized in this region, and this sample set offered the opportunity to begin to define the locally important serotypes. Ad distributions are geographically variable, and serotype is clinically relevant because it determines vaccine efficacy and correlates strongly with both symptomology and epidemiological patterns. Serotypes were determined using several well-validated multiplex PCR protocols culled from the literature and supplemented with a few novel primer sets designed to identify rare types. The isolates included the common species B1 serotypes Ad3 and Ad7, the less common Ad11, and common species C serotypes Ad1, Ad2, and Ad5. Three isolates of the rare species B1 serotype Ad16, and two isolates that appear to be either variant Ad16s or closely related novel serotypes, were also identified. These variants, as well as the Ad11 isolates, were only firmly identified at the serotype level through the use of secondary methods (restriction enzyme analysis and sequencing, respectively), though serial PCR was able to identify them as rare serotypes of species B (not Ad3, 7, or 21). The primary method used in this study, serial multiplex PCR, is very rapid and requires very little equipment. The work presented here demonstrates the ease with which clinically relevant strain information about respiratory Ad isolates can be obtained without cumbersome neutralization tests.

INTRODUCTION

Adenoviruses (Ads) are a diverse group of double-stranded DNA viruses responsible for a wide variety of human ailments (25). Ads are categorized by species (AdsA, B1, B2, C, D, E, and F), further by serotype (Ads1-51), and even further by genome type. These distinctions correlate strongly with both clinical presentation and epidemiological character (27), and the same groups are highly clustered on the basis of genetic homology (10). The most clinically important Ad species are those associated with febrile respiratory disease (AdsB1, C, and E) since these can cause widespread outbreaks with severe symptomologies, including viral pneumonia and death (16, 18, 23, 26).

Ad3 and Ad7 (both species B1 serotypes) cause large disseminated outbreaks that include both adults and children, and are sometimes seen together (15, 26). These two serotypes are often responsible for the most severe respiratory symptomologies associated with adenoviruses (11, 17). Outbreaks of Ad3 and Ad7 appear worldwide and have been documented in Japan (18), Korea (12), the United Kingdom (6), the United States (4, 8), South America (16), China (19) and elsewhere.

Military recruit populations in the United States regularly suffer from high rates of acute respiratory disease caused by AdE serotype 4, and AdB serotypes 3, 7, 14, and 21. In these populations, outbreaks can encompass the majority of the population (25, 26). AdB also includes the less common serotypes 11, 16, 34, and 35, all of which are associated, at least occasionally, with respiratory disease.

The AdC species includes serotypes 1, 2, 5, and 6. These are generally associated with common endemic or sporadic respiratory disease in children (25), a pattern sometimes shared

with Ad3 (8). AdC serotypes are also associated with a wide variety of illness in immunocompromised patients (1). Serosurveys suggest that virtually all people are exposed to these Ads during childhood (14, 24). They can be retained in an asymptomatic carriage state until at least young adulthood (9), and may be actively shed long after symptomatic infection (8). These patterns strongly suggest a reason for the endemic nature of these Ads, and also point to the problems that may be associated with assigning causality based upon viral detection alone. In fact, one controlled study of 18,000 children and infants found that many healthy children in non-epidemic situations test positive for AdC by culture, though sick children were significantly more likely to test positive (3).

Clearly, serotype information is medically relevant. Serotype correlates with the severity and symptomology of disease, as well as with epidemiological characteristics (11, 25, 28). Knowledge of circulating serotypes can also predict if developed vaccines might be useful. The worst epidemics, often caused by new genome types (as defined by restriction enzyme analysis), may occur in areas where the causal serotype was rare in recent times (23).

Adenovirus serotype is traditionally determined through neutralization tests, in which antibodies raised against specific serotypes are used to suppress cytopathic effect in tissue culture assays. These tests are technically demanding, lengthy (2 weeks), and require expensive and difficult-to-obtain antiserum. They also require active viral culture and associated biosafety measures, which are not available in most clinical settings (1).

Not surprisingly, serotype correlates very well with sequence polymorphisms in the genes coding for the primary antigenic determinants, including both the hexon coat protein and the receptor-binding fiber protein. This correlation allowed the development and validation of several multiplex PCR assays (1, 31, 32) which, when used together, are capable of

discriminating all of the common respiratory Ad serotypes. This method of testing is far less demanding than traditional neutralization, and only relies on commonly available and inexpensive equipment and reagents.

Multiplex PCRs are also capable of revealing coinfections of multiple Ad serotypes (32) whereas neutralization, by nature of its design, can only reveal one dominant serotype. Our own recent work with PCR-based identification has shown that coinfections may be common in specific populations or environments (Vora, Metzgar, manuscript in review). Some of these coinfections include species C Ads, which may be asymptomatic carriage state infections retained from initial infection in childhood (9). Others, however, are clearly coinfections of multiple virulent adenoviral serotypes that do not typically result in long-lasting latent infections, such as the serotypes known to cause outbreaks in military recruits (serotypes 3, 4, 7, and 21) (Vora, Metzgar, manuscript in review). The clinical implications of these coinfections are not yet clear, but the availability and increasing popularity of molecular tests capable of recognizing multiple coinfecting serotypes should permit elucidation of their impact.

We used serial multiplex PCR to identify the serotypes of adenovirus associated with influenza-like illness in Egypt, a country with previously poor documentation of circulating Ad strains.

MATERIALS AND METHODS

Sample collection and processing. Throat swab samples (n=88) were collected as part of routine influenza surveillance from outpatients with influenza-like illness (ILI) at Monira General Hospital (Cairo), Kitchener General Hospital (Cairo), and Alexandria Fever Hospital (Alexandria), all in Egypt. Samples were collected during the period January 1999 - June 2002. Patients were recruited if their symptoms fit the World Health Organization definition of ILI or if, in the judgement of the physician, the patient was suffering from an ILI. All but two had fevers exceeding 38°C. Cough, headache, muscle ache, runny nose, fatigue, sore throat, and chills were all common (>50%). Some patients (>30%) also experienced vomiting. Samples were tested for influenza and adenovirus at the Naval Medical Research Unit 3 facility in Cairo, Egypt. All samples used in this study tested negative for influenza. Ad was identified by immunofluorescence after growth on MRC-5 or H292 tissue culture cell lines. Isolates were obtained from patients aged 0–55 years, with about two thirds of the isolates coming from infants, children, and adolescents (<18 years).

Infected tissue culture fluid (ITCF) was collected and frozen at Naval Medical Research Unit 3 and sent frozen on dry ice to the Naval Health Research Center (NHRC) for further testing. Two-hundred microliter aliquots of ITCF samples were extracted using the Epicenter MasterPure Complete DNA and RNA Purification Kit (Epicenter, Madison, Wis) as per the directions for saliva. Extracts were stored frozen at -20°C, and remaining ITCF was refrozen and stored at -80°C when available. While the strains identified herein were tested as tissue culture amplified isolates, the same techniques are fully applicable to original patient specimens. Our laboratory routinely uses PCR on original specimens as a means of identifying Ad among

military recruit populations, and we have thoroughly tested and validated this method using culture as the gold standard. We have found PCR and culture to be approximately equal in sensitivity; hence, any sample that can be cultured and tested as an ITCF can also be tested by direct extraction and PCR of the patient sample (either nasal wash or throat swab). It should be noted that many coinfections, which are often quite biased in titer, may yield amplicons for only one strain in original samples.

We have noticed that direct extraction of high-titer ITCF is subject to cross-contamination, and exceptional sterility in processing methods must be maintained. This problem was never encountered with lower titer original patient specimens. Furthermore, high-titer samples can yield very weak cross-reaction bands with the AdB serotype-specific multiplex primers (see Fig. 1 and Results and Discussion: Methodology). It is recommended that ITCF (grown virus) be diluted 1:100 into water before extraction and PCR with these methods (particularly the AdB serotype multiplex), and that Ad3, Ad7, and/or Ad21 controls be grown, extracted, and processed by PCR in parallel with clinical isolates for ease in identification of serotype-specific bands.

PCR. All PCRs were performed using iCyclers (BioRad, Hercules, Calif). PCR reactions were set up in a clean room then transferred to a separate room for addition of the extracted templates to avoid contamination. PCR, gel electrophoresis, and analysis were performed in a third room. Most of the primers used here were taken from the existing literature, and these were exhaustively tested against neutralization results in previous papers. Primers from existing papers included the Ad universal primers (32), the primers for the split species-specific multiplex testing for species sets (B and E) and (A, C, D, and F) (32), the species B serotype-specific multiplex primers testing for Ad3, Ad7, and Ad21 (31), the species C serotype-specific multiplex primers

testing for Ad1, Ad2, Ad5, and Ad6 (1), and the Ad4-specific primers (13). We also developed our own primers to distinguish rare members of species B not covered by the existing tests (the results of these were only accepted when confirmed by a second method; see Results and Discussion). These novel primers, targeting polymorphisms in the hexon gene, are shown in Table 1. For purposes of further discussion, the reactions using these primers will be termed "novel monoplex PCRs".

PCRs were carried out as shown in the flow chart (Fig. 2). We found that the original species-specific multiplex (32) often resulted in nonspecific bands arising from the species C-specific primers, which could potentially generate false-positives for AdB or AdE. When this multiplex was divided in two, testing seperately for the sets (B and E) and (A, C, D and F), these potential misidentifications were obviated. Furthermore, small changes were applied to some of the protocols in order to re-optimize the reactions using the specific polymerases and reagents available in our laboratory. While the flow chart depicts only the minimum number of steps needed to identify respiratory Ad serotypes, we tested all controls and all samples with all PCRs to make sure there were no cross-reactions. Reactions were performed as follows:

Multiplex PCRs were performed using the QIAGEN Multiplex Kit (QIAGEN, Valencia, Calif). Reactions contained 0.5X Q-solution, 1X Multiplex buffer, 0.2 μM concentration of each primer (Integrated DNA Technologies, Coralville, Iowa), and 2 μl of extracted sample in 25-μl aqueous reactions.

Monoplex PCRs contained 1X Q-solution (QIAGEN), 1X PCR buffer (Promega, Madison, Wis), 0.6 μM concentration of each primer (Integrated DNA Technologies), 0.8 mM concentration of each deoxynucleoside triphosphate (Promega), 3 mM concentration of $MgCl_2$

(Promega), 1.25 U of *Taq* polymerase (Promega), and 2 µl of extracted sample in 25 µl aqueous reactions.

Efforts were made to find a set of common cycling conditions that would allow similar processing of samples through the various reactions. These efforts were generally unsuccessful, and we found that it was much more effective to optimize each multi- and monoplex PCR independently. All cycles included a 10-min final extension at 72°C and a final hold at 4°C. Otherwise, reactions were cycled as shown in Table 2.

All products were mixed 5:1 with loading dye (Sigma Aldrich, St. Louis, Mo) and run for 90 min at 125 V on 1.5% agarose (Sigma Aldrich) gels with ethidium bromide (Sigma Aldrich) . Standard 100-bp DNA ladders were used as a reference (New England Biolabs, Beverly, Mass). We also used "product ladders" for the multiplexes, generated by balanced combination of all Ad types targeted by the multiplex. Gels were visualized and recorded by photography on a UV light box.

Due to the occasional appearance of faint, gray bands resulting from mispriming between closely related B-species serotypes, products were recorded as positive when a band of "normal" intensity was observed in the expected size range. Normal intensity was defined as being within the range observed among expected bands amplified from positive control strains (see Fig. 1 and Results and Discussion: Methodology).

Primer design. Novel monoplex primer sets were designed by J.W. to target serotype-specific sequences in the hexon gene of AdB serotypes that were not included in the existing multiplexes. Primers sequences were chosen using an alignment of all adenoviral hexon sequences available in GenBank (http://www.ncbi.nlm.nih.gov/Entrez/index.html). The general

rarity of these strains makes multiplexing unnecessary, since they need only be used when a sample tests negative by the multiplex for common serotypes.

In the case of the novel monoplex reactions we did not have a positive control for the targeted strains, and therefore could not test the specificity or sensitivity of the assays. Positive results for Ad16 were cross-validated by the size of the AdB-specific band in the species-specific multiplex. This band size is unique among AdBs and is known to distinguish Ad16 (32). The samples testing positive for Ad11 also tested positive for Ad14 despite three mismatched bases in one of the Ad14 primers (as predicted by the GenBank sequences for these serotypes). Therefore, we sequenced positive PCR products from the Ad11-specific PCRs and compared them to the available sequences in GenBank using the Basic Local Alignment Search Tool family of programs (http://www.ncbi.nih.gov/BLAST/). Sequences clearly showed these strains were Ad11 and not Ad14.

Controls. Control strains and sources are listed in Table 3. All positive controls were previously grown in the College of American Pathologists-certified NHRC Virology laboratory, and all were originally serotyped by either the Centers for Disease Control and Prevention (CDC), the American Type Culture Collection (ATCC), or by microneutralization (20) in the NHRC laboratory.

RESULTS AND DISCUSSION

Epidemiology. Results are shown in Fig. 3. The results show a surprisingly high rate of AdB1 infection relative to AdC when compared with general large-scale survey analyses (28). However, the high rate of AdB1 in this population may simply reflect the circumstances—these Ads are known to cause the most severe respiratory symptoms (17), and Egyptians are reluctant

to seek medical assistance for conditions that are not severe (S.Y., personal observation). Similar relative rates of AdB and AdC were seen among hospitalized pneumonia patients in Korea (12).

Among AdBs, Ad3 was by far the most common serotype identified from patients with ILI in Egypt, followed by Ad7. Four isolates of the less common Ad11 were identified, as were three isolates of the rare Ad16 and two apparent Ad16 variants (Ad16*). The two Ad16 variants could only be subtyped within B1 by restriction enzyme analysis (A. K., manuscript in preparation), because they did not yield a product with Ad16-specific primers or show the characteristic Ad16-specific band in the AdB/AdE multiplex (they yielded normal AdB amplicons). These appear to be unique strains and will be further analyzed. The Ad11 isolates tested positive with both the novel Ad11 and Ad14 monoplex PCRs, and were firmly identified as Ad11 by sequencing of the amplicon from the Ad11 primer set.

For the most part, these rates reflect those seen in other countries, including the United Kingdom (6), the United States (3, 8), Korea (12), China (19), and the world as a whole (28). As is the case here, Ad3 and Ad7 usually dominate AdB respiratory disease. The relative dominance of Ad3 and Ad7, the two serotypes implicated in the worst outbreaks (11), tends to vary both temporally and spatially, perhaps because of the protective effect of mass immunity imposed by recently circulating strains (23). The number of non-3 or -7 AdBs is considerably higher in this sample set than in other similar studies (6, 8), but the sample size is small enough that this could simply be chance.

The presence of Ad16 is notable because this strain is rarely even mentioned in the literature. Two of the three patients with typical Ad16 infections were infants (<1 year), one male and one female, while the other was a 30-year-old female. All were collected in Alexandria but were spread over an 8-month period and do not appear to be epidemiologically related.

Isolates grew on both MRC-5 and H292 cell lines. Since this serotype is not the focus of specific surveillance and neutralization tests are often not extended to theoretically rare serotypes, it may be that this serotype is often underdiagnosed. Ad16 may also be uniquely important in this region. The prototype Ad16 was previously isolated in the Middle East, from a conjunctivitis/trachoma patient in Saudi Arabia (2, 21). Ad16 has been occasionally reported outside the Middle East in broad surveys, associated with symptomologies including respiratory disease (4, 30). In these cases, isolation rates of this virus were too low (<1%) for strong measures of association with specific symptoms and controlled surveys have found similar rates of isolation from ill patients and healthy controls (4). In contrast to this, one striking report from Arkansas reported two separate deaths, one adolescent and one adult, one from Reye's syndrome-like symptoms and the second a viral pneumonia, both linked to Ad16 infection in otherwise physically healthy patients (21). In these cases the virus was isolated from parenteral organs related directly to the symptoms (spleen and lung, respectively). Given the low rates of isolation of Ad16 in general, these two deaths are good reason to observe and track this serotype. If the two Ad16 variants (Ad16*) seen here are also considered, the rates of Ad16 in this population are extremely high.

A similar situation is found in the case of Ad11. Ad11 is associated with acute hemorrhagic cystitis, both naturally (22) and, sometimes fatally, as the result of the virus hitchhiking with transplanted kidneys and bone marrow (5) . It was also associated with a fatal pneumonia in an immunocompromised patient (29). Like Ad16, Ad11 is rarely isolated in respiratory illness surveys (8) and strong causal correlations are generally lacking. However, serosurveys show that Ad11 is generally rare (7), so associations with specific symptomologies are suggestive of a causal etiology (30).

Certainly, future studies of respiratory Ads should not exclude these rare but potentially devastating serotypes methodologically, and it should be kept in mind that an understanding of the local distribution of Ads demands the use of maximally inclusive methods.

AdC serotypes, which are generally endemic among children, were also found. Identified strains included Ads 1, 2, and 5. These are common, and their occurrence is expected given the age distribution of the patients from which they were collected. It is important to remember that these, unlike species B1 adenoviruses, usually generate long-lasting latent infections characterized by persistent intermittent excretion (3, 8). Therefore, positive results do not necessarily reflect a causal link between the viruses and recent symptoms.

The data collected during this study did not reveal any strong temporal or spatial trends within Egypt during the study period. Table 4A shows the distribution of serotypes for each year studied, while Table 4B shows the seperate distributions of serotypes collected in Cairo and Alexandria.

This study revealed many Ad coinfections (see Fig. 3). Many of these involve AdB and AdC, while others involve multiple serotypes of AdB. The rarity of reported coinfections in the literature may well be the result of methodology - neutralization tests are comparative and qualitative, and are inherently biased towards identification of a single serotype. Multiplex PCR, on the other hand, can amplify minority targets as effectively as majority targets, and is biased in the other direction - towards identification of serotypes even in cases where one coinfecting serotype is present at much lower titer than the other.

It can be seen by comparison of the monoinfection and coinfection charts (Fig. 3) that the common coinfections tend to involve the most common serotypes. This suggests that the composition of coinfections may be determined by chance associations of multiple serotypes,

rather than by unique features of specific pairings. The clinical significance of adenoviral coinfections, like the significance of Ad11 and Ad16, can only be understood through further surveillance and analysis.

Methodology. Serial multiplex PCR, supported by a few novel PCR tests designed to identify generally rare strains, provides a rapid and inclusive method of serotype identification. Of 88 strains, the method successfully identified a species in all cases and a serotype in 82 cases. Four of the six untypeable isolates were identified as either Ad11 or Ad14 by the primary method, and further identified as Ad11 by sequencing of the PCR amplicon. The two remaining untypeable isolates now have been studied further through restriction enzyme analysis and appear to be variants of Ad16 (Ad16*) (A. K., manuscript in preparation). In all cases except one (a possible coinfection identified as Ad7 + Ad16*), positive serotype identifications were supported by species results, including cases for which multiple serotypes (coinfections) were observed.

Results in general were clear and conclusive. All control strains and negative controls yielded results consistent with expectations, strengthening the already intensive validations of the literature-derived multiplex PCRs used here. The novel monoplex primers developed and used to determine the serotype of otherwise unidentifiable isolates were less exact, but they served their purpose. Identifications of three of the Ad16 isolates found here were readily validated with reference to Ad16-specific signatures from the species-specific (AdB and AdE) multiplex. The other apparent Ad16s (Ad16*) matched some known Ad16 molecular signatures but not others. This may well be because so little molecular data exists for Ad16, and hence the range of molecular signatures found among Ad16s is not yet well defined. Identifications of Ad11 were

easily confirmed with sequencing, which is available to any PCR-enabled laboratory at low cost through commercial sequencing services. Initial efforts to develop Ad34-specific primers were unsuccessful, but further work would certainly offer a method for this as well. All of the novel tests used here would benefit from further development and optimization, but the rarity of these strains in commonly surveyed environments may render such efforts unnecessary. The important thing is that these infections were diagnosed with enough resolution to reveal the impact of these potentially underestimated serotypes.

The AdB serotype-specific test relies on rare polymorphisms to distinguish these very closely related strains. We found that the primers used in this test are capable of producing weak (gray) "shadow bands" in nonspecific cross-reactions when used to test some high-titer ITCF isolates. This can be seen in two of the cases shown in the Ad3, 7, and 21 portion of Fig. 1 (Ad7 field strain (FS) b and Ad16 FSa). Our laboratory recently saw similar bands in control samples used in other studies. In both of the cases noted in Fig. 1, monoplex tests for the serotypes suggested by the shadow bands gave negative results. Therefore, we adopted a criterion for positive results that demands a clear band, within the range seen among correct bands for positive control samples. Further efforts toward clinical validation of these methods suggested that these shadow bands can be avoided by 10- or 100-fold dilution of ITCF samples prior to extraction. In any case, these shadow bands are readily distinguishable from the much brighter bands resulting from specific amplification.

The inclusiveness of this method, extending to both rare serotypes and coinfections of multiple serotypes, makes it an attractive and accessible alternative to classic culture and neutralization methods. Furthermore, this method serves to identify not only serotype, but also

presence/absence and species, and in that sense offers a complete method of analysis, one that can be performed on either grown virus or on original patient specimens.

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REFERENCES

1. **Adhikary, A. K., T. Inada, U. Banik, J. Numaga, & N. Okabe** (2004). Identification of subgenus C adenoviruses by fiber-based multiplex PCR. J. Clin. Microbiol, **42(2):**670-673.
2. **Bell, S. D., D. E. McComb, E. S. Murray, R. S. Chang, & J. C. Snyder** (1959). Adenoviruses isolated from Saudi Arabia. I. Epidemiologic features. Am. J. Trop. Med. Hyg, **8:**492-500.
3. **Brandt, C. D., H. W. Kim, B. C. Jeffries, G. Pyles, E. E. Christmas, J. L. Reid, R. M. Chanock, & R. H. Parrott (**1972). Infections in 18,000 infants and children in a controlled study of respiratory tract disease. II. Variation in adenovirus infections by year and season. Am. J. Epidemiol, **95:**218-227.
4. **Brandt, C. D., H. W. Kim, A. J. Vargosko, B. C. Jeffries, J. O. Arrobio, B. Rindge, R. H. Parrott, & R. M. Chanock** (1969). Infections in 18,000 infants and children in a controlled study of respiratory tract disease. I. Adenovirus pathogenicity in relation to serologic type and illness syndrome. Am. J. Epidemiol, **90:**484-500.
5. **Carrigan, D. R.** (1997). Adenovirus infections in immunocompromised patients. Am. J. Med, **102(3A):**71-74.
6. **Cooper, R. J., R. Hallett, A. B. Tullo, & P. E. Klapper** (2000). The epidemiology of adenovirus infections in Greater Manchester, UK 1982-96. Epidemiol. Infect, **125:**333-345.
7. **D'Ambrosio, E., N. Del Grosso, A. Chicca, & M. Midulla** (1982). Neutralizing antibodies against 33 human adenoviruses in normal children in Rome. J. Hyg (Lond), **89:**155-161.
8. **Fox, J. P., C. E. Hall, & M. K. Cooney (**1977). The Seattle Virus Watch VII. Observations of adenovirus infections. Am. J. Epidemiol, **105:**362-386.
9. **Garnett, C.T., D. Erdman, W. Xu, & L.R. Gooding** (2002). Prevalence and quantitation of species C adenovirus DNA in human mucosal lymphocytes. J. Virol, **76:**10608-10616.
10. **Green, M., J. K. Mackay, W. S. M. Wold, and P. Rigden** (1979). Thirty-one human adenovirus serotypes (Ad1-Ad31) form five groups (A-E) based upon DNA genome homologies. Virology, **93:**481-492.
11. **Hierholzer, J.** (1995). Adenoviruses. p. 169-188. *In* E. H. Lennette, D. A. Lennette, and E. T. Lennette (eds.), Diagnostic procedures for viral, rickettsial and chlamydial infections, 7th ed. American Public Health Association, Washington, D.C.

12. **Hong, J. Y., H. J. Lee, P. A. Piedra, E. H. Choi, K. H. Park, Y. Y. Koh, & W. S. Kim (**2001). Lower respiratory tract infections due to adenovirus in hospitalized Korean children: epidemiology, clinical features, and prognosis. Clin. Infect. Dis, **32:**1423-1429.

13. **Houng, H. S., S. Liang, C. M. Chen, J. Keith, M. Echavarria, J. L. Sanchez, S. A. Kolavic, D. W. Vaughn, & L. N. Binn** (2002). Rapid type-specific diagnosis of adenovirus type 4 infection using a hexon-based quantitative fluorogenic PCR. Diagn. Microbiol. Infect. Dis, **42**:227-236.

14. **Jordan, W. S. Jr.** (1958). Occurrence of adenovirus infections in civilian populations. Arch. Intern. Med, **101:**54-59.

15. **Kajon, A. E., A. S. Mistchenko, C. Videla, M. Hortal, G. Wadell, & L. F. Avendano** (1996). Molecular epidemiology of adenovirus acute lower respiratory infections of children in the south cone of South America (1991-1994). J. Med. Virol, **48:**151-156.

16. **Kajon, A. E., S. A. R. Portes, W. A. de Mello, J. P. Nascimento, & M. M. Siqueira** (1999). Genome type analysis of Brazilian adenovirus strains of serotypes 1, 2, 3, 5, and 7 collected between 1976 and 1995. J. Med. Virol, **58:**408-412.

17. **Kajon, A., and G. Wadell** (1994). Genome analysis of South American adenovirus strains of serotype 7 collected over a 7-year period. J. Clin. Microbiol, **32:**2321-2323.

18. **Kim, Y. J., J. Y. Hong, H. J. Lee, S. H. Shin, Y. K. Kim, T. Inada, M. Hashido, & P. A. Piedra** (2003). Genome type analysis of adenovirus types 3 and 7 isolated during successive outbreaks of lower respiratory tract infections in children. J. Clin. Microbiol, **41:**4594-4599.

19. **Li, Q. G., Q. J. Zheng, Y. H. Liu, & G. Wadell** (1996). Molecular epidemiology of adenovirus types 3 and 7 isolated from children with pneumonia in Beijing. J. Med. Virol, **49:**170-177.

20. **Malasig, M. D., P. R. Goswami, L. K. Crawford-Miksza, D. P. Schnurr, & G. C. Gray** (2001). Simplified microneutralization test for serotyping adenovirus isolates. J. Clin. Microbiol, **39:**2984-2986.

21. **Morgan, P. N., E. B. Moses, E. P. Fody, & A. L. Barron** 1984. Association of adenovirus type 16 with Reye's-syndrome-like illness and pneumonia. South. Med. J, **77:**827-830.

22. **Mufson, M. A., & R. B. Belshe** (1976). A review of adenoviruses in the etiology of acute hemorrhagic cystitis. J. Urol, **115:**191-194.

23. **Noda, M., T. Yoshida, T. Sakeguchi, Y. Ikeda, K. Yamaoka, & T. Ogino** (2002). Molecular and epidemiological analyses of human adenovirus type 7 strains isolated from the 1995 nationwide outbreak in Japan. J. Clin. Microbiol, **40:**140-145.

24. **Potter, C. W. & W. I. H. Shedden** (1963). The distribution of adenovirus antibodies in normal children. J. Hyg. (Lond), **61:**155-160.

25. **Rubin, B. A. (**1993). Clinical picture and epidemiology of adenovirus infections. Acta Microbiol. Hung, **40:**303-323.

26. **Ryan, M. A., G. C. Gray, B. Smith, J. A. McKeehan, A. W. Hawksworth, & M. D. Malasig** (2002). Large epidemic of respiratory illness due to adenovirus types 7 and 3 in healthy young adults. Clin. Infect. Dis, **34:**577-582.

27. **Sambrook, J., M. Sleigh, J. A. Engler, & T. R. Broker** (1980). The evolution of the adenoviral genome. Ann. N. Y. Acad. Sci, **354:**426-452.

28. **Schmitz, H., R. Wigand, & W. Heinrich** (1983). Worldwide epidemiology of human adenovirus infections. Am. J. Epidemiol, **117:**455-466.

29. **Siegel, F. P., S. H. Dikman, R. B. Urayota, & E. J. Bottone** (1981). Fatal disseminated adenovirus type 11 pneumonia in an agammaglobulinemic patient. Am. J. Med, **71:**1062-1067.

30. **Wold, W. S. M., J. K. Mackay, P. Rigden, & M. Green** (1979). Analysis of human cancer DNAs for DNA sequences of human adenovirus serotypes 3, 7, 11, 14, 16, and 21 in Group B[1]. Cancer Res, **39:**3479-3484.

31. **Xu, W. & D. D. Erdman** (2001). Type-specific identification of human adenovirus 3, 7, and 21 by a multiplex PCR assay. J. Med. Virol, **64:**537-542.

32. **Xu, W., M. C. McDonough, & D. D. Erdman** (2000). Species-specific identification of human adenoviruses by a multiplex PCR assay. J. Clin. Microbiol, **38(11):**4114-4120.

TABLE 1. Novel primers developed for AdB serotypes 11, 14, 16, and 35.

Name	Primer sequence
Ad11-F	gaagtttcagatgaagaaagta
Ad11-R	aggacttaagtttgttttctgc
Ad35-F	gatttcagctgaaaacgaatct
Ad35-R	gtctggtgtttccaaacctaca
Ad14-F	aaatgctaatcttggacagcagtc
Ad14-R	agccgtccagtggaaaacagtagt
Ad16-F	gctggcattctggacacggagttg
Ad16-R	cctcatgaaggccgtatatccag

TABLE 2. Cycling conditions.[a]

PCR step	Cycles (#)	Universal	Species-specific	B serotypes	C serotypes	Serotype 4	Novel monoplexes
Initial denaturation	1	5:00(95°C)	15:00(95°C)	5:00(95°C)	5:00(95°C)	5:00(95°C)	5:00(95°C)
Denaturation	35	1:00(94°C)	0:30(94°C)	1:00(94°C)	1:00(94°C)	0:40(94°C)	1:00(94°C)
Annealing		1:00(52°C)	1:30(52°C)	1:00(56°C)	1:00(47°C)	0:40(53°C)	1:00(53°C)
Extension		1:30(72°C)	2:00(72°C)	1:00(72°C)	2:00(72°C)	1:30(72°C)	1:30(72°C)

[a] All conditions are shown as time (temperature), with time shown as minutes:seconds.

TABLE 3. Control strains

Control strain	Source	ID by PCR
Ad12 (Huic[a])	CDC[b]	AdA
Ad3 (GB, VR-3[a])	ATCC	AdB, Ad3
Ad7 (Gomen[a])	ATCC[b]	AdB, Ad7
Ad1 (ws 2/5/91[a])	CDC[b]	AdC, Ad1
Ad2	CDC	AdC, Ad2
Ad5 (VR-1516[a])	ATCC	AdC, Ad5
Ad8 (ws 2/18/91[a])	CDC[b]	AdD
Ad4 (NIH 5/66[a])	CDC[b]	AdE, Ad4
Ad40 (Dugan[a])	CDC[b]	AdF
Influenza A	ATCC	none
Influenza B	ATCC	none
hMPV[c]	NHRC	none
Healthy patient	NHRC	none
Water	Sigma	none
VTM[d] (blank)	Remel	none

[a] Strain designations as listed by provider.

[b] These strains generously provided by Dean Erdman and CDC. Other strains were obtained from CDC Biologicals or ATCC, or were contained within archives from NHRC surveillance.

[c] human metapneumovirus

[d] VTM, viral transport medium (Remel, Lenexa, KS).

Table 4A. Serotype distribution by year of isolation.

	Ad3	Ad7	Ad16	Ad16*	Ad11	Ad1	Ad2	Ad5
1999	4	3	0	0	0	2	0	1
2000	17	3	1	1	3	3	2	0
2001	34	9	2	1	0	7	1	1
2002	5	2	0	0	1	1	0	0
Total	60	17	3	2	4	13	3	2

Table 4B. Serotype distribution by city of isolation.

	Ad3	Ad7	Ad16	Ad16*	Ad11	Ad1	Ad2	Ad5
Cairo	10	5	0	0	0	4	0	1
Alexandria	50	12	3	2	4	9	3	1
Total	60	17	3	2	4	13	3	2

FIG. 3. Serotype and coinfection distributions of 88 adenovirus isolates from Egypt. Serotypes are grouped by species and further by serotype. All single-serotype counts include isolates from individuals with coinfections. Colors in coinfection frequency bars represent the paired components of the coinfections. Ad16* and the coinfection of Ad7 and Ad16* refer to isolates that gave serotype-specific positive results for Ad16 by either serotype-specific PCR or restriction enzyme analysis, but yielded only a normal AdB species amplicon with the species-specific primers. Since Ad16 is expected to yield a uniquely sized amplicon for this serotype (see Fig. 1 and Results and Discussion), these results represent ambiguous Ad16 positive results. These are either sequence variants of Ad16 or new serotypes most closely related to Ad16. VTM, viral transport medium.

FIG. 1. Representative gels showing results with the AdB and E multiplex primers and the Ad3, 7, and 21 multiplex primers for 20 samples. FS, field strain (isolate from Egypt); CS, control strain (previously serotyped isolate); VTM, viral transport medium (negative control). Ad4/7 ladder and Ad3/7/21 ladder are amplicons from control strain mixtures, termed product ladders, designed to enable easy band identification.

FIG. 2. Flow chart representing the series of PCRs used to identify strains in this study.

Figure 1

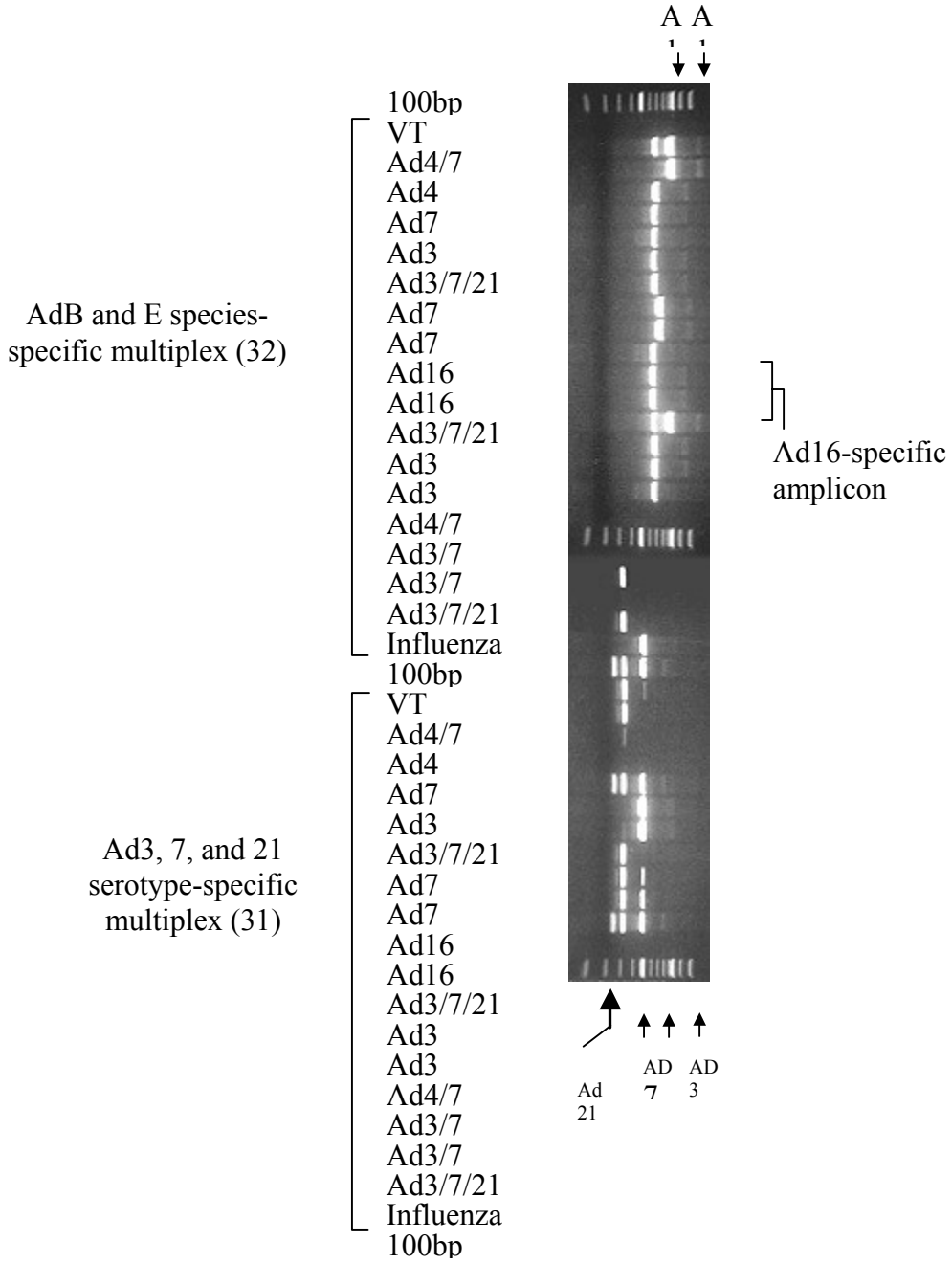

A A
100bp
VT
Ad4/7
Ad4
Ad7
Ad3
Ad3/7/21
Ad7
Ad7
Ad16
Ad16
Ad3/7/21
Ad3
Ad3
Ad4/7
Ad3/7
Ad3/7
Ad3/7/21
Influenza
100bp
VT
Ad4/7
Ad4
Ad7
Ad3
Ad3/7/21
Ad7
Ad7
Ad16
Ad16
Ad3/7/21
Ad3
Ad3
Ad4/7
Ad3/7
Ad3/7
Ad3/7/21
Influenza
100bp
AdB and E species-specific multiplex (32)
Ad3, 7, and 21 serotype-specific multiplex (31)
Ad16-specific amplicon
Ad 21
AD 7
AD 3

Figure 3

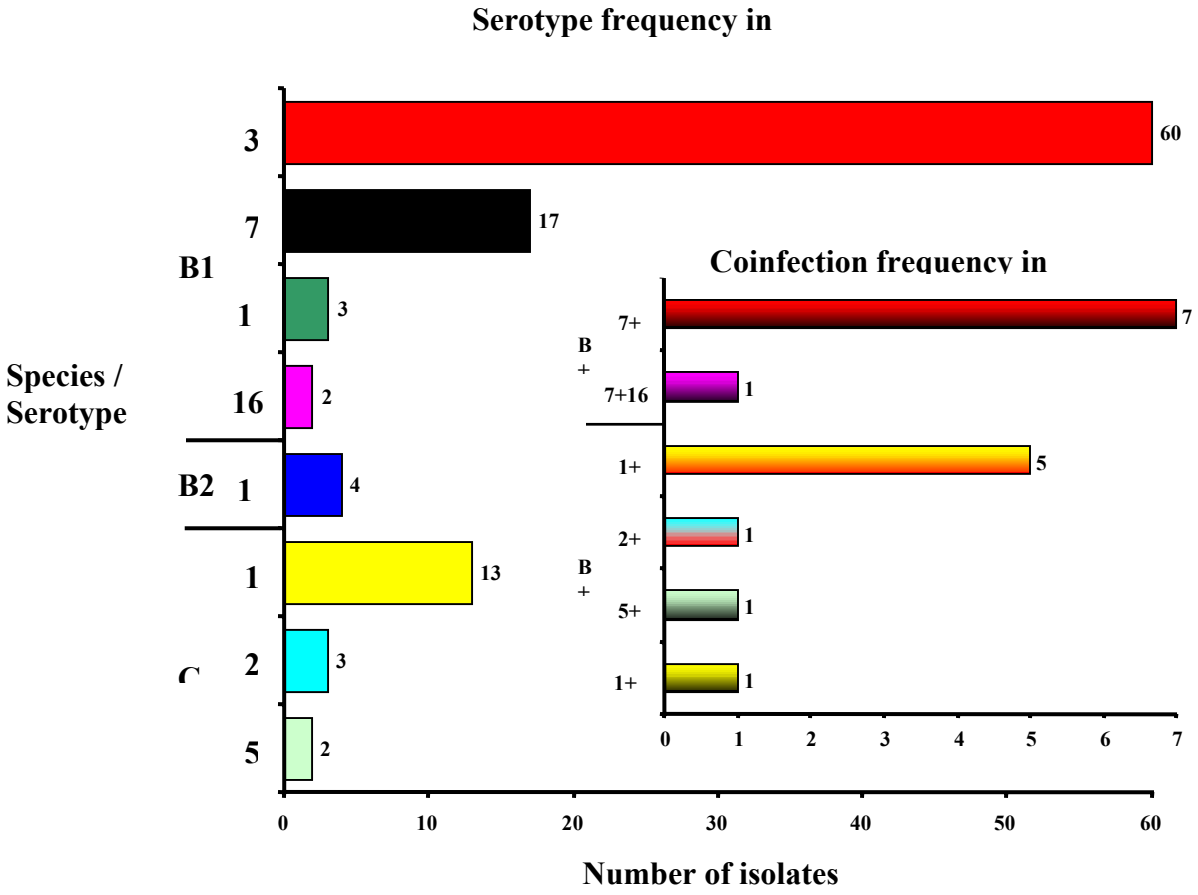
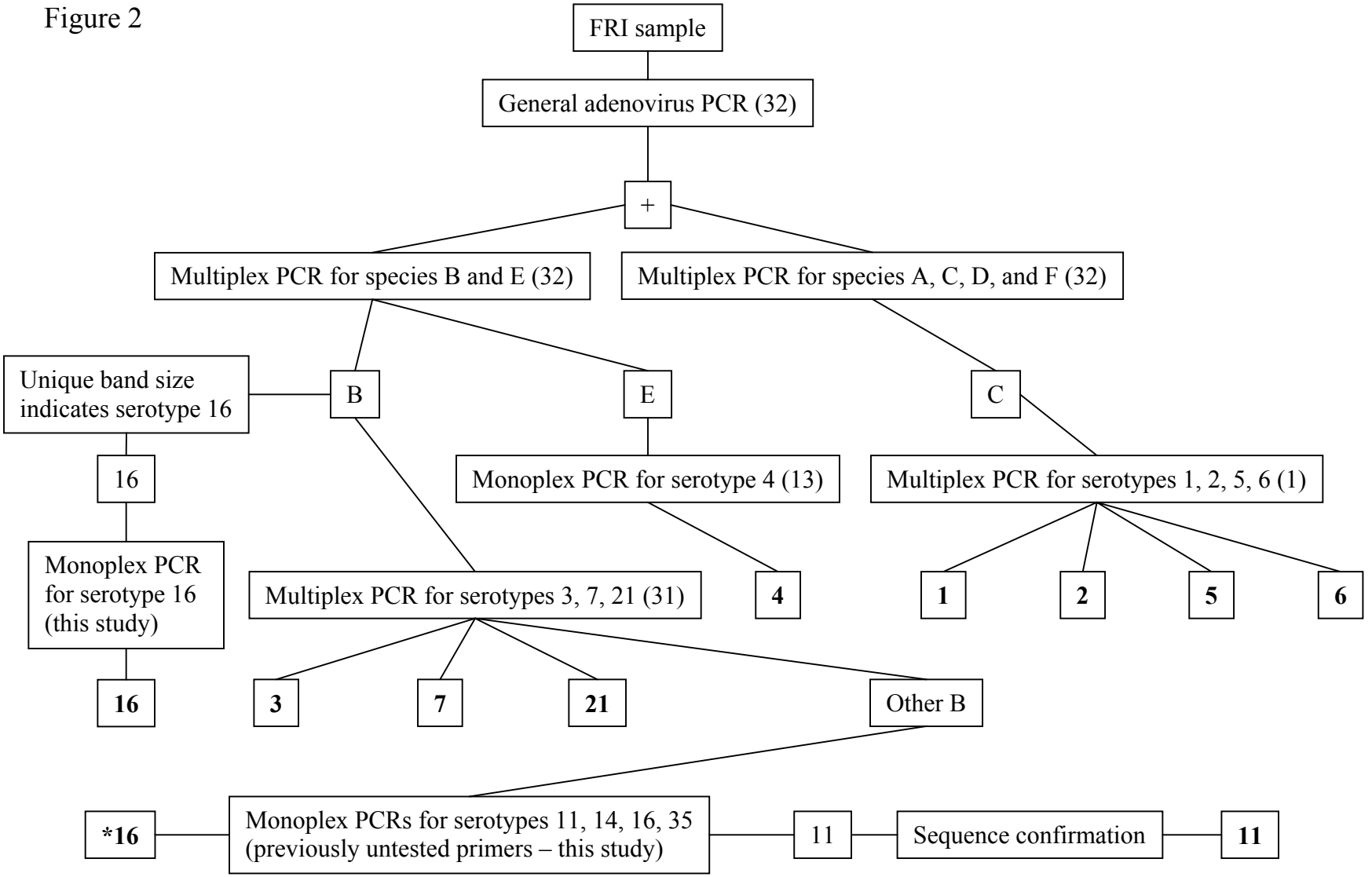

Serotype frequency in
Species / Serotype
3
7
B1
1
16
B2
1
1
C
2
5
60
17
3
2
4
13
3
2
Coinfection frequency in
7+
B+
7+16
1+
2+
B+
5+
1+
7
1
5
1
1
1
0
10
20
30
40
50
60
0
1
2
3
4
5
6
7
Number of isolates

Figure 2


FRI sample
General adenovirus PCR (32)
+
Multiplex PCR for species B and E (32)
Multiplex PCR for species A, C, D, and F (32)
Unique band size indicates serotype 16
B
E
C
16
Monoplex PCR for serotype 4 (13)
Multiplex PCR for serotypes 1, 2, 5, 6 (1)
Monoplex PCR for serotype 16 (this study)
Multiplex PCR for serotypes 3, 7, 21 (31)
4
1
2
5
6
16
3
7
21
Other B
*16
Monoplex PCRs for serotypes 11, 14, 16, 35 (previously untested primers – this study)
11
Sequence confirmation
11

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14. ABSTRACT (maximum 200 words)

Eighty-eight adenovirus (Ad) isolates and associated clinical data were collected from walk-in patients with influenza-like illness in Egypt during routine influenza surveillance. Ad diversity has not been well characterized in this region, and this sample set offered the opportunity to begin to define the locally important serotypes. Ad distributions are geographically variable, and serotype is clinically relevant because it determines vaccine efficacy and correlates strongly with both symptomology and epidemiological patterns. Serotypes were determined using several well-validated multiplex PCR protocols culled from the literature and supplemented with a few novel primer sets designed to identify rare types. The isolates were dominated by the common species B1 serotypes Ad3 and Ad7, the less common Ad11, and common species C serotypes Ad1, Ad2, and Ad5. Three isolates of the rare species B1 serotype Ad16, and three isolates that appear to be either variant Ad16s or closely related novel serotypes, were also identified. Two of these variants, as well as the Ad11 isolates, were only firmly identified at the serotype level through the use of secondary methods (restriction enzyme analysis and sequencing, respectively), though serial PCR was able to identify them as rare serotypes of species B (that is, not Ad3, 7, or 21). The method used to type these isolates, serial multiplex PCR, is very rapid and requires very little equipment. We use this survey to demonstrate the ease with which clinically relevant strain information about respiratory Ad isolates can be obtained without cumbersome neutralization tests.

15. SUBJECT TERMS
Middle East, Arabia, adenovirus, serotype, multiplex PCR, epidemiology

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